Bioaugmentation of activated sludge with *Achromobacter denitrificans* PR1 for enhancing the biotransformation of sulfamethoxazole and its human conjugates in real wastewater: Kinetic tests and modelling

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Abstract

Achromobacter denitrificans PR1 has previously shown potential to degrade the antibiotic sulfamethoxazole, whereby sulfamethoxazole biotransformation was stimulated in the presence of biogenic substrates. This study examined the biotransformation kinetics of sulfamethoxazole and its two main conjugates, N_4-acetyl-SMX and SMX-N_1-Glucuronide, by activated sludge and activated sludge bioaugmented with *A. denitrificans* PR1. SMX biotransformation under both anoxic and aerobic conditions was tested, with and without the addition of acetate as growth substrate, to understand the range of applicable conditions for bioaugmentation purposes. Biological process models, such as the pseudo-first order kinetic and cometabolic models, were also applied and, following the estimation of kinetic parameters, could well describe data measured in bioaugmented and non-bioaugmented AS batch experiments under various test conditions. Experimental and modelling results suggest that (i) retransformation of the two conjugates to SMX in AS occurred under both aerobic and anoxic conditions, and (ii) biotransformation kinetics of SMX can vary significantly depending on redox conditions, e.g., SMX was biotransformed by AS only under aerobic conditions. Notably, SMX biotransformation was significantly enhanced when PR1 was bioaugmented in AS. Addition of acetate as biogenic substrate is not neccessary, as PR1 was capable of enhancing the SMX biotransformation by using the carbon sources present in wastewater. Overall, bioaugmentation by means of *A. denitrificans* PR1 could be a viable strategy for enhancing SMX removal in AS wastewater treatment plants (WWTPs).

Keywords: antibiotics, cometabolism, N_4-acetyl-SMX, SMX-N_1-Glucuronide, modelling, retransformation

1. Introduction

The intensive use of antibiotics for human and veterinary therapy has led to their continuous discharge, also in the form of conjugates, in the environment. WWTPs are not designed to remove these and other xenobiotic chemicals, and discharge of treated effluents into the environment has been a major concern due to the risk of a worldwide dispersal of antibiotic resistance genes [1].

Amongst antibiotics, sulfamethoxazole (SMX) is one of the most widely used synthetic sulfonamides worldwide. SMX enters WWTPs via human excretion in the forms of unchanged SMX (15-25% of the excreted dose) as well as the conjugated forms N_4-acetyl-SMX (Ac-SMX) (> 40%) and SMX-N_1-Glucuronide (SMX-Glu) (9-15%) [2]. The two human conjugates have been detected in wastewater influent and effluent, and were observed to rapidly deconjugate during wastewater treatment [3,4] which was considered to likely explain the reported 'negative removal' of SMX in wastewater treatment [3,5]. This suggests the importance of investigating the retransformation of the two major human conjugates to parent SMX, in order to explain the reported differences in removal efficiencies in WWTPs [6,7].

SMX removal has been shown to vary greatly, i.e. from negative (-138%) to very high (>90%) [1] in full-scale WWTPs, and with variability in SMX biotransformation kinetics. SMX was also shown to be not readily biodegradable during the 28-day test period in a closed bottle test [8].

Biotransformation has been recognized as the major elimination mechanism of SMX and its conjugates during biological treatment of domestic wastewater, with minor contribution of sorption onto sludge (due to the polar nature of these compounds). Overall, literature reports of inconsistent and incomplete SMX elimination suggest that novel technologies/strategies would be required if more stringent discharge limits for SMX and other antibiotics are enforced. Bioaugmentation can be an alternative WWTP operational strategy to enable or enhance xenobiotics removal by inoculating specialized degrading bacteria [9]. Despite the fact that bioaugmentation has been studied for years in wastewater treatment to reinforce biological processes, few studies have tested the use of bioaugmentation for enhancing the removal of xenobiotics, e.g. 17β-estradiol [10], estradiol [11], fungicides [12]. With respect to antibiotics, bioaugmentation resulted in limited SMX removal when applying *Microbacterium* sp. strain BR1 in full-scale membrane bioreactors [13], except for SMX concentrations far higher than the ones normally found in municipal wastewater.

Previously, we showed that a pure culture of *Achromobacter denitrificans PR1* could exhibit faster biotransformation kinetics (up to two to three orders of magnitude higher) of SMX compared to AS alone [14], even at the low SMX concentrations typical of wastewater effluents. Given its ability to degrade SMX in the presence and/or absence of other additional carbon sources (acetate and succinate) at environmentally relevant concentrations (typical of e.g., wastewater effluents), the strain likely has potential for treating SMX in wastewater upon bioaugmentation. Therefore, the overall objective of this work was to investigate whether PR1 can enhance SMX biotransformation kinetics when bioaugmented to AS with real wastewater feed. Specifically, we (i) investigated the effect of redox conditions, i.e. aerobic and anoxic conditions, on the transformation rates of targeted compounds; (ii) assessed the potential influence of retransformation processes of the two main human conjugates, i.e. Ac-SMX and SMX-Glu, on the fate of sulfamethoxazole under the testing conditions; and (iii) evaluated the need for supplementation with a biogenic substrate (e.g. acetate) or whether the availability of carbon sources in wastewater could serve as biogenic substrates to achieve a sufficiently interesting kinetic for SMX removal upon bioaugmentation of AS with PR1. Modelling the fate of xenobiotics in WWTPs can be a useful tool to understand their removal mechanisms, predict and reduce their emissions with treated effluent through process optimization. Specifically, the Activated Sludge Modelling framework for Xenobiotics (ASM-X), has been previously used to predict the fate of SMX in biological treatment systems [7] and to identify factors (influent concentration of conjugates, solid residence time) possibly explaining the variability in SMX removal efficiencies [15]. In this context, suitable mathematical models were developed to examine the metabolic mechanism and predict kinetics of SMX and human conjugates biotransformation upon bioaugmentation of *A. denitrificans* PR1 into AS.

2. Materials and methods

2.1. Chemicals and reagents

Reagent grade (purity ≥ 99%) SMX was purchased from Sigma-Aldrich. Ac-SMX, SMX-Glu and isotopically labelled Ac-SMX-d4, SMX-d4-Glu, SMX-d4 were obtained from Toronto Research Chemicals, Inc. (TRC, Canada). Individual stock standard solutions were prepared on a weight basis in methanol and stored at -20ºC. HPLC-grade methanol was supplied by Merck (Darmstadt, Germany).

2.2. *Laboratory-scale experiments - Bioaugmentation of AS with A. denitrificans* **PR1**

2.2.1. Culture media

Bacterial inoculum was grown in mineral medium B, supplemented with ammonium phosphate at concentration of 400 mM (designated as MMBN), as previously described by [16]. The cells were harvested by centrifugation (7000 x g for 10 min at 20ºC using a Sigma® 4-16KS centrifuge), and rinsed three times with fresh MMBN medium to remove the trace amount of SMX remaining from the culture medium before augmenting to the reactors to get an initial cell suspension concentration of approximately 0.05-0.06 $mg_{biomass}$ L^{-1}.

2.2.2. Batch tests

Biotransformation of SMX and the two main human conjugates by AS and bioaugmented AS was assessed in a series of batch experiments in 1 L jacketed glass reactors. Dried compressed atmospheric air or pure nitrogen were continuously sparged by a diffuser placed at the bottom of each reactor to create aerobic or anoxic conditions, respectively. Temperature was controlled at 20°C using an external recirculating bath and pH was monitored and maintained between 7.0-7.4 by the addition of HCl (0.2 M) or NaOH (0.2 M), using pH controllers (HI8711, Hanna Instruments, US) with dual set point.

For all experiments, primary effluent wastewater and AS (from a Modified Ludzack Ettinger system) collected from the Chelas WWTP (Lisbon, Portugal) were used. More information about Chelas WWTP is provided in Section S2 and Table S3, Supplementary Information (SI). AS and primary effluent were seeded to the 1 L glass reactors at an initial biomass concentration of approximately 3 gTSS L^{-1} for all the experiments. Overall, four types of batch tests were performed: (i) abiotic control tests; (ii) sorption tests; (iii) bioaugmenation tests; (iv) nitrification inhibtion tests. The testing conditions are presented in Table 1. All the tests were performed in duplicate, except for the anoxic bioaugmented AS test (An2, Table 1), the control 1 and the allylthiourea (ATU) nitrification inhibition tests.

Abiotic control test (control 1)

The goal of this experiment was to determine the contribution of abiotic removal mechanisms (stripping, sorption onto reactor walls and equipment, and abiotic chemical reactions). In this test, the 1 L-glass-reactor

was filled with Milli-Q water that was spiked with the three compounds, e.g. SMX, Ac-SMX and SMX-Glu at the concentrations of 10 µg L^{-1}, 15 µg L^{-1} and 15 µg L^{-1}, respectively. The experiment lasted 6 hours.

Sorption tests (control 2)

Sodium azide (NaN_3) is a well know respiration inhibitor used for negative control tests in AS studies. A wide range of concentrations from 0.5 to 720 $mg_{azide}g_{TSS}^{-1}$ were used in previous studies for this purpose [17]. In this test, a concentration of ~ 650 $mg_{azide}g_{TSS}^{-1}$ was used to inhibit AS activity. SMX, Ac-SMX and SMX-Glu were spiked into the reactors at the initial concentrations of 5 µg.L^{-1}, 10 µg.L^{-1} and 10 µg.L^{-1}, respectively. The tests were performed in duplicate.

Bioaugmentation tests

The goal of these tests was to assess biotranformation of the targeted compounds with non-bioaugmented and bioaugmented AS with *A. denitrificans* PR1. Batch experiments were performed during 12 to 14 hours under aerobic and anoxic conditions. In aerobic tests, the influence of a biogenic substrate on SMX biotransformation was assessed by adding acetate at an initial concentration of ~ 137 to 152 $mg_{COD}L^{-1}$ that is similar to the level of soluble COD typically found in many activated sludge WWTPs.

In anoxic batch tests, reactors were supplemented with an initial nitrate concentration of 80 mg NO_3-N L^{-1} in the form of KNO_3. Aqueous stock solutions of SMX and the two target conjugates were spiked to obtain an initial concentration of approximately 5 µg L^{-1} and 10 µg L^{-1}, respectively.

Table 1. Overview of the different tested conditions in the bioaugmented and non-bioaugmented AS experiments

		Batch	Feed	AS (gTSS L^{-1})	*A.denitrificans* **PR1** ($g_{biomass}$ L^{-1})	Acetate ($mg_{COD} L^{-}$)	NaN_3 ($mg_{azide} g_{TSS}^{-1}$)	ATU (mg L^{-1})
Control	Control 1	C1	MilliQ-water	-	-	-		
	Control 2	C2	WW*	~ 3	-	-	~ 650	
Nitrification-inhibition		ATU	WW*	~ 3	-	-		30
		Without ATU	WW*	~ 3	-	-	-	-
Bioaugmentation tests	Aerobic	A1	WW*	~ 3	0	0		
		A2	WW*	~ 3	0	137-152		
		A3	WW*	~ 3	~ 0.05-0.06	0		
		A4	WW*	~ 3	~ 0.05-0.06	137-152		
	Anoxic	An1	WW*	~ 3	0	0		
		An2	WW*	~ 3	~ 0.05-0.06	0		

WW*: wastewater from the effluent of a primary sedimentation tank was centrifuged at 10000 x g for 15 min at 4°C, and then filtered through Whatman® Glass microfiber filters, pore size 1.2 µm binder free, Grade GF/C before feeding to the reactors.

Nitrification inhibition tests

To determine the contribution of ammonia oxidizing bacteria and heterotrophs to the SMX biotransformation in AS communities, biomass was inactivated using ATU at 30 mg L^{-1} [18], a copper chelator that depletes

copper ions from the active center of ammonia monooxygenases (AMO), therefore inhibiting ammonia oxidizing activity.

2.3. Sample preparation and analytical procedures

Samples collected along the tests were centrifuged for 5 min at 8000 xg, followed by syringe filtration through 0.2 μm cellulose Whatman filters and stored at -20ºC prior to analysis of soluble chemicals.

The acetate concentrations were determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Total and volatile suspended solids (TSS, VSS) were determined according to Standard Methods (APHA, 1995). Ammonium, nitrate and nitrite concentrations were measured using a segmented flow analyzer through the Skalar San++ system. Samples were also analyzied for soluble COD (sCOD) using HACH-lange test kits and a DR2800 spectrophotometer (HACH, Germany).

Analysis of SMX and the two human conjugates was performed on a high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. Detailed descriptions of the sample preparation and analytical methods used are provided as Section S1 (SI).

2.4. Determination of kinetic parameters-modelling approach

2.4.1. Modelling assumptions

In this study, we hypothesized that (i) only retransformation of the two conjugates, e.g. Ac-SMXl and SMX-Glu, will occur through deconjugation to form the parent compound SMX and that (ii) the dissolved compounds are the only biodegradable fractions. Thus, the biotransformation of SMX includes two processes: (i) formation of SMX due to the retransformation (deconjugation) of Ac-SMX and SMX-Glu; (ii) simultaneous elimination of SMX.

2.4.2. Model implementation and estimation of parameters

In this study, the biotransformation rate of the three target compounds was calibrated using the ASM-X modelling framework [7,15,19].

Deconjugation of the two human conjugates (Ac-SMX and SMX-Glu) to form the parent compound SMX is described by a pseudo-first order kinetic model (Table 2, process (1) for aerobic and process (9) for anoxic removal), thus allowing the estimation of the biotransformation rate coefficients, e.g. $k_{Dec,Ox}$ or $k_{Dec,Ax}$ (L $gTSS^{-1}$ d^{-1}).

For the biotransformation of SMX under aerobic conditions, both pseudo-first order and cometabolic models were implemented to test which one could appropriately predict SMX biotransformation (Table 2). The cometabolic biotransformation model [19] consisted of two biotransformation rates: the enhanced rate in the presence (q_{bio}, L d^{-1} g^{-1}) and the pseudo-first order rate in the absence (k_{bio}, L d^{-1} g^{-1}) of growth substrates. Accordingly, biotransformation kinetics of the cometabolic substrate (e.g. micropollutants) depend on the readily biodegradable growth substrates, S_S (mgCOD L^{-1}). S_S was determined as the difference between soluble COD (sCOD, measured during the experiments) and soluble inert COD (S_I – calculated according to [20]). The initial S_S concentration of the pre-clarified municipal wastewater used in this study ranged between 41 and 128 mgCOD L^{-1}. Parameters that could not be identified through model calibration to experimental results (i.e. heterotrophic yields Y_H, substrate affinity constant K_S) were adopted from literature [21]. Concentration profiles of acetate, expressed as sCOD, were used to calibrate the maximum specific growth rate of heterotrophs μ_H (Table S5 and Fig. S1, SI). The estimated parameters included: (i) biotransformation rate constants of the AS ($k_{bio,AS}$) and the bioaugmented strain ($k_{bio,PR1}$) in the absence of primary substrate; and (ii) the cometabolic biotransformation rate constants of the AS ($q_{bio,AS}$) and the bioaugmented strain ($q_{bio,PR1}$) in the presence of the primary substrates. Each batch test was designed to determine a specific kinetic constant and is described in Table 3. The model was implemented in Aquasim 2.1d [22] and the embedded secant method was used for parameter estimation.

In our study, experimental data from A1, A4, An1 and An2 tests were used for the model calibration and estimation of the biotranformation rate constants of SMX and the two human conjugates by AS and *A. denitrificans* PR1 under aerobic and anoxic conditions (Table 3). More details on the model calibration procedure are presented in Supplementary Information (section S3).

188 *2.4.3. Model validation*

189 Two different sets of experimental results (A2 and A3) were used to validate the cometabolic kinetic models
190 calibrated with the data sets of A1 and A4.

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Table 2. Stoichiometric (Gujer) matrix of the ASM-X for processes of parent compound retransformation, biotransformation and the alternative cometabolic biotransformation model. Parameters and state variables for determination of micropollutants kinetics are described in the main text.

Processes i → j process ↓	C_{LI}	C_{CJ}	S_s	X_{AS}	X_{PR1}*	Process rate
Pseudo-first order kinetics – Aerobic processes						
(1) *Parent compound formation due to retransformation of human*	F	-1		*		$\frac{k_{Dec} C_{CJ} X_{AS}}{1 + K_D X_{AS}}$
(2) Pseudo-first order kinetics – biotransformation transformation of parent compound C_{LI} by AS	-1			*		$\frac{k_{bio\_AS} C_{LI} X_{AS}}{1 + K_D X_{AS}}$
(3) Pseudo-first order kinetics – biotransformation of parent compound C_{LI} by the bioaugmentation strain, i.e. A. denitrificans *PR1*	-1				*	$\frac{k_{bio\_PR1} C_{LI} X_{PR1}}{1 + K_D X_{PR1}}$
Cometabolic model – Aerobic processes						
4) Cometabolic biotransformation of C_{LI} by AS	-1			*		$\frac{(q_{bio\_AS} \frac{S_S}{S_S + K_S} + k_{bio\_AS}) C_{LI} X_{AS}}{1 + K_D X_{AS}}$
(5) Cometabolic enhancement biotransformation of C_{LI} by the bio-augmentation strain, i.e. A. denitrificans *strain PR1*					*	$\frac{(q_{bio\_PR1} \frac{S_S}{S_S + K_S} + k_{bio\_PR1}) C_{LI} X_{PR1}}{1 + K_D X_{AS}}$
(6) Aerobic growth			*		*	$\mu_H \frac{S_S}{S_S + K_S} X_H$

Pseudo first order kinetics – Anoxic processes				
(7) Parent compound formation due to retransformation of human conjugates C_{CJ}	F	-1	*	$\frac{k_{Dec} C_{CJ} X_{AS}}{1 + K_D X_{AS}}$
(8) Biotransformation of parent compound C_{LI} by AS	-1		*	$\frac{k_{bio\_AS} C_{LI} X_{AS}}{1 + K_D X_{AS}}$
(9) Biotransformation of parent compound C_{LI} by the bio-augmentation strain, i.e. A. denitrificans *PR1*	-1		*	$\frac{k_{bio\_PR1} C_{LI} X_{PR1}}{1 + K_D X_{PR1}}$

*Due to short duration of the batch experiment and low S/X ratio, negligible biomass growth was assumed.

F = ratio between molecular mass of parent compound and metabolite undergoing deconjugation.

S_S: primary substrate concentration (e.g., organic matter or acetate in some of these experiments, expressed as readily soluble biodegradable COD) considering a co-substrate (gCOD L^{-1}).

C_{LI} and C_{CJ} : the aqueous concentrations of the parent compound and the human conjugates undergoing deconjugation to the parent compound, respectively (μg L^{-1}).

k_{Dec}: retransformation rate constant of deconjugation of the human conjugates to parent compound (L $gTSS^{-1}$ d^{-1}).

k_{bio_AS} : is the reaction rate coefficient of biotransformation of parent compound (L $gTSS^{-1}$ d^{-1}) by AS.

k_{bio_PR1}: is the reaction rate coefficient of biotransformation of parent compound (L $gTSS^{-1}$ d^{-1}) by the bioaugmented *A. denitrificans* strain PR1.

K_S : half-saturation coefficient for S_S

K_D: sorption coefficient (0.256 L $gbiomass^{-1}$ for SMX - [23]). The values are not available for N_4-acetyl-SMX and SMX-N_1-Glucuronide, and were thefore assumed to be equal to 0.

203 X_{PR1} or X_{AS} : biomass concentration of bio-augmented strain *A. denitrificans* or AS, expressed in gTSS L^{-1};

204 X_H is expressed in gCOD L^{-1} by assuming biomass-to-COD ratio of 0.75

Table 3. Model calibration and parameter estimation procedures for the batch tests performed under aerobic and anoxic conditions

Batch	Goal	Process used	Input parameters	Estimated parameters
A1 (non-bioaugmented)	Model calibration	Process (1)		$k_{dec\_N4\_Ox}$, $k_{dec\_Glu\_Ox}$
		Process (2)		$k_{bio\_AS\_Ox}$
		Process (1), (4) and (6)	$k_{dec\_N4\_Ox}$, $k_{dec\_Glu\_Ox}$, $k_{bio\_AS\_Ox}$	$q_{bio\_AS\_Ox}$
A4 (bioaugmented)	Model calibration	Process (3)		$k_{bio\_PR1\_Ox}$ **
		Process (1), (4), (5) and (6)	$k_{dec\_N4\_Ox}$, $k_{dec\_Glu\_Ox}$, $k_{bio\_AS\_Ox}$, $q_{bio\_AS\_Ox}$, $k_{bio\_PR1\_Ox}$	$q_{bio\_PR1\_Ox}$
A2 (non-bioaugmented)	Model validation	Process (1), (4) and (6)	$k_{dec\_N4\_Ox}$, $k_{dec\_Glu\_Ox}$, $k_{bio\_AS\_Ox}$, $q_{bio\_AS\_Ox}$	None
A3 (bioaugmented)	Model validation	Process (1), (4), (5) and (6)	$k_{dec\_N4\_Ox}$, $k_{dec\_Glu\_Ox}$, $k_{bio\_AS\_Ox}$, $q_{bio\_AS\_Ox}$, $k_{bio\_PR1\_Ox}$, $q_{bio\_PR1\_Ox}$	None
An1 (non-bioaugmented)	Model calibration	Process (7)		$k_{dec\_N4\_Ax}$, $k_{dec\_Glu\_Ax}$
		Process (8)	$k_{dec\_N4\_Ax}$, $k_{dec\_Glu\_Ax}$	$k_{bio\_AS\_Ax}$
An2 (bioaugmented)	Model calibration	Process (7), (8) and (9)	$k_{dec\_N4\_Ax}$, $k_{dec\_Glu\_Ax}$, $k_{bio\_AS\_Ax}$	$k_{bio\_PR1\_Ax}$

** $k_{bio\_PR1\_Ox}$ was determined in our previous study [14], from the test with the pure culture (i.e. *A.denitrificans* PR1 biodegradation test) conducted in mineral medium supplemented with SMX as the only substrate.

3. Results and discussions

3.1. Abiotic and sorption processes

Fig. 1 shows the evolution of SMX, Ac-SMX and SMX-Glu concentrations over the test period of 6 h in control test 1, revealing 1.8%, 11.4% and 11.8% removal for Ac-SMX, SMX-Glu and SMX, respectively. This suggests that abiotic processes had minor contribution to the removal of the tested compounds, in agreement with previous studies [24].

To investigate sorption to AS, sodium azide (NaN_3) was used to inhibit the aerobic respiration and suppress the microbial activity of the AS (control 2). The results showed that Ac-SMX and SMX-Glu were transformed concomitantly with an increase in SMX concentration (Fig. 1), indicating that the retransformation of the parent SMX from the two human conjugates occurred even with inactivated biomass, likely via extracellular enzymes. This is in agreement with previous studies for other conjugates [25]. In terms of mass balance, supposing that all the human conjugates were converted back to SMX, there was approx. 0.02 µmol SMX formed after 4.5 hours, while there was a removal of approx. 0.02 µmol of the two human conjugates. Therefore, no SMX removal was observed in the presence of NaN_3 (control 2). Since sodium azide was present at concentrations previously observed to be sufficient to inhibit the fraction of aerobic biomass [26], biotransformation of the two human conjugates could be due to the activity of facultative anaerobic bacteria, which was not sufficiently inhibited by the addition of NaN_3.

Due to the impossibility of determining the partitioning coefficient for SMX, the sorption fraction was assessed considering the sorption coefficient K_D obtained from previous literature. A K_D value of 0.256 L $gTSS^{-1}$ [23] was chosen as the tests in this study were performed with fresh AS and real wastewater, which was representative of the real WWTPs where the K_D was obtained. At circumneutral pH typical of activated sludge systems, SMX is predominantly speciated as an anion (pK_a = 5.7). Possibly due to repulsion with negatively charged slugde particles, sorption of SMX has been generally found to be limited (K_d < 0.4 L g^{-1}) but not negligible. Notably, the K_d value used is in agreement with other determinations in activated sludge (see, e.g. [7], [27], [23]). Sorption of the two human conjugates onto

AS was not considered in these experiments as no reference values of sorption coefficient were reported. Indeed, the pH of the mixed liquor in the tests was between 7.0-7.4, which is well above the pK_a of SMX-Glu and Ac-SMX (pK_{a2} = 2.7 and 5.6, respectively). Under these experimental conditions, Ac-SMX and SMX-Glu exist predominantly or completely as negatively charged species in the aqueous phase. Hence, negligible sorption of Ac-SMX and SMX-Glu was assumed due to their high solubility and polar nature.

3.2. Biotransformation of SMX, SMX-Glu and Ac-SMX in bioaugmented and non-bioaugmented AS tests

Aerobic batch experiments (A1-A4)

In general, biotransformation of the two human conjugates was almost complete for all the batch tests performed under aerobic conditions. This is in agreement with previous studies, showing >85% removal of the two conjugates in laboratory-scale [3] and full-scale [4] AS processes, or even fully eliminated in a pilot membrane bioreactor [28]. In the aerobic non-bioaugmented reactors (A1, A2), an increase in SMX concentration was observed in the first 4–6 hours followed by a slow decrease during the rest of the tests when biotransformation of the two human conjugates was complete (Fig. 2a and 2b). Negative SMX removal was observed, i.e. -43.1% and -63.8%, for the AS in tests A1 and A2, respectively. The decrease in Ac-SMX and SMX-Glu concentrations corresponded to increases in SMX concentrations (Fig. 2a, 2b), strongly suggesting that the two human conjugates deconjugated rapidly to form the parent compound SMX under aerobic conditions. There is relatively limited knowledge on the environmental fate and behavior of the conjugated pharmaceuticals, but these conjugates can undergo deconjugation reactions where deconjugation enzymes are present, with cleavage of the conjugated moiety, resulting in the formation of the parent pharmaceuticals [15,29].

For bioaugmentation of AS with PR1, experimental results obtained in the two batches A3 and A4, show a comparably high rate of SMX biotransformation in the first 4 hours, followed by a relatively lower SMX removal rate after the growth substrates were completed (Fig. 2c and 2d). Similar SMX removal was observed in A3 and A4 after 12 hours, i.e. 92.5 ± 1.0% and 89.4 ± 1.5%, respectively.

263 Concomitantly, complete removal of the SMX-Glu and Ac-SMX in the first 4 hours of the test was
264 observed, which supposedly was converted back to SMX (Fig. 2c and 2d) as suggested by other studies
265 [3,15].

266 *Relative contribution of heterotrophs and ammonia oxidizing bacteria*

267 Batch tests performed in the presence of ATU showed no removal of ammonium and no formation of
268 nitrate (Fig. 3b), suggesting nitrification was completely suppressed There was 42 % removal of SMX
269 after 12 hours (Fig. 3a). No appreciable differences were observed in the removal efficiency of SMX
270 with and without ATU (Fig. 3a), suggesting negligible contribution of AOB to the biotransformation of
271 SMX. Also, for all the remaining aerobic batch tests (A1, A2, A3, and A4) performed, no ammonium
272 removal and no nitrate formation could be observed (Fig. S2) during the testing periods, confirming that
273 no nitrifying activity occurred in the tested AS. Even though biotransformation of SMX in AS was
274 previously shown to correlate with both nitrifying activity [30,31] and heterotrophic bacteria [32,33],
275 no appreciable differences were observed in the removal efficiency of SMX with and without ATU (Fig.
276 3a) in our study, which could be due to the fact that (i) the SMX biotransformation rate by heterotrophic
277 aerobic degradation was reported to be much faster compared to autotrophic nitrification (e.g. $k_{bio,\,h}$ =
278 0.09 L/g_{VSS} d vs. $k_{bio,\,a}$ = 0.01 L/g_{VSS} d) [32]; and (ii) the possible higher abundance of heterotrophs
279 compared to nitrifiers. Thus, heterotrophs seem to be the dominant organisms responsible for the
280 biotransformation of SMX in the current study and the cometabolic model applied for all the batch tests
281 could be based on only organic carbon (i.e. readily biodegradable substrates and supplemental acetate)
282 as the primary substrates.

283 *Effect of acetate addition on the biotransformation of SMX*

284 We previously showed that the presence of a biogenic substrate, e.g. acetate or succinate, provided for
285 an 8-fold increase of SMX biotransformation kinetics by PR1 [14]. In the current study, the initial
286 concentration of acetate was approximately 137 to 152 mg COD L^{-1}, supplemented with preclarified
287 wastewater to (i) the bioaugmented reactor (A4) (Fig. S8b) as a biogenic substrate to enhance the
288 kinetics of SMX biotransformation by PR1, and (ii) the non-bioaugmented reactor (A2) (Fig. S8a) as a

control for comparison purposes. Upon bioaugmentation of AS with PR1 (reactor A3, Fig. 2c), with only wastewater (no acetate addition), SMX was biotransformed steadily and almost completely without any lag phase. In addition, the profiles of SMX in the tests fed with acetate were comparable to the one in the tests without acetate (Fig. S8a (A1 versus A2) and S8b (A3 versus A4)). From these observations, we hypothesized that (i) PR1 could use other available carbon sources present in wastewater as biogenic substrates to enhance the SMX biotransformation kinetics and the addition of acetate is unnecessary for the bioaugmentation with PR1; (ii) there is no enhancement effect due to acetate addition on the biotransformation of SMX by AS when fed with real wastewater. These hypotheses were also justified with the modelling results in section 3.3 and 3.4.

Anoxic experiements

Pure culture biotransformation tests had previously shown that the *A.denitrificans* PR1 is capable of biotransformation of SMX under both aerobic [14] and anoxic conditions (data not shown). Hence the extent of SMX removal under anoxic conditions was also assessed and compared to those obtained in aerobic conditions.

In the two anoxic batch tests with non-bioaugmented (An1) and bioaugmented AS (An2), most of the two human conjugates were removed in the first 6 hours (Fig. 4a and 4b),which is in agreement with Stadler et al. 2015 [3] that observed >90% of Ac-SMX and SMX-Glu removals under anoxic condition.

For the non-bioaugmented reactor An1, the consumption of nitrate (Fig. 4c) revealed denitrifying activity, while no net SMX removal could be observed (Fig. 4a) overall. SMX concentration increased in the first 6 hours simultaneously with the deconjugation of the two human conjugates, and remained constant for the rest of the experiment. This is opposed to what was observed in denitrifying AS [7] and denitrifying MBBR sludge [34,35]. In contrast, in the bioaugmented reactor (An2), a slight decrease in SMX concentration was observed after the retransformations of the two human conjugates had completed (after 4 hours) (Fig. 4b). This suggests a biotransformation of SMX associated with the activity of the bioaugmented strain PR1, but at a rather slow rate.

3.3. Model-based assessment of biotransformation kinetics

Experimental data obtained in the batches A1, A4 and An1, An2 was used for the estimation of the biotransformation rate constants for AS and the *A. denitrificans* PR1 under aerobic and anoxic conditions. Predicted dissolved concentration profiles of SMX and its two human conjugates during batch experiments are compared with measured data and shown in Fig. 2a, 2c, Fig. 4a and 4b. The estimated parameters are summarized in Table 4. The model predictions were evaluated using the R-squared (R^2) coefficient, shown in Fig. S3-S5, and summarized in Table S4 (SI). Confidence intervals were obtained by the estimation of standard deviations for a level of confidence of 95% (Fig. S3-S5, SI).

Kinetics of deconjugation

Deconjugation kinetics of the two conjugates could be described with pseudo-first order kinetics, in processes for aerobic (1) and anoxic (9) conditions. For the conjugated Ac-SMX, fitting of measured data resulted in k_{Dec} values of 8.9 ± 0.53 L $gTSS^{-1}$ d^{-1} for aerobic conditions, which was almost 2-fold higher than anoxic conditions (5.30 ± 0.21 L $gTSS^{-1}$ d^{-1}) (Table 4). These data agree well with values reported in literature for k_{Dec} of 5.9-7.6 L $gTSS^{-1}$ d^{-1} [7,36] under aerobic conditions or 7.9 L $gTSS^{-1}$ d^{-1} [7] under anoxic conditions. No difference in the rate constants of SMX-Glu under aerobic and anoxic conditions was obtained (4.76 ± 0.4 and 4.74 ± 0.31 L $gTSS^{-1}$ d^{-1}, respectively). No data for the biotransformation rate coefficients of SMX-Glu were available in literature for comparison. Good agreement between experimental data and model simulations for Ac-SMX and SMX-Glu was shown, as confirmed by high R^2 coefficients (≥0.98), indicating that pseudo-first order equations describe well the biotransformation kinetics of the two human conjugates. These results suggest that (i) deconjugation rate constants are well above 1 L $gTSS^{-1}$ d^{-1}, thus indicating high degradability for conjugates; and (ii) deconjugation kinetics depend on redox conditions for Ac-SMX only, being faster under aerobic conditions.

Kinetics of SMX biotransformation under aerobic conditions

The removal of SMX under aerobic conditions was predicted using different mathematical models for comparision (i) a pseudo-first order kinetic model; and (ii) cometabolic models. Fig. 2 and Fig. S6 (SI) compare predicted and measured concentrations of SMX in the batches A1-A4, using a cometabolic model and a pseudo first order model, respectively. According to the data plotted in these figures, the prediction of SMX biotransformation was significantly improved by adopting the cometabolic model (R^2 ranged from 0.79 to 0.99, Table S4, SI) compared to pseudo-first order biotransformation model (R^2 ranged from 0.044 to 0.94). These results: (i) show that the cometabolic model was able to consistently describe the experimental data, with measured concentrations that fall well within the 95% confidence interval (Fig. S3, S4, SI), making the cometabolic model the relevant choice for description of the removal of SMX; (ii) support our hypothesis of the deconjugation of Ac-SMX and SMX-Glu results in the formation of parent compound SMX, which likely explains the previously observed variability/negative SMX removal efficiencies in biological treatment. In this study, we also simulated two other scenarios for the fate of SMX, i.e.: (i) biotransformation of the two human conjugates leading to the formation of a compound different from SMX (model simulations presented as blue dashed lines in Fig. 2 and 4); and (ii) all of the Ac-SMX and SMX-Glu are converted back to parent SMX, but no SMX is biodegraded, and the model simulation is presented as black dashed lines in Fig. 2 and Fig. 4. However, the model simulations in these scenarios were far different from the respective observed concentrations of SMX in all the batch experiments (Fig. 2 and 4), indicating that neither situation is applicable in the current study.

According to our simulation results, a SMX biotransformation rate constant $k_{bio,AS}$ of 0.47 ± 0.03 L $gTSS^{-1}$ d^{-1} and a cometabolic biotransformation rate constant q_{bio_AS} of 7.97 ± 0.51 L $gTSS^{-1}$ d^{-1} (Table 4) were obtained by AS under aerobic conditions. Previous studies reported $k_{bio,SMX}$ of 0.14 – 0.41 L $gTSS^{-1}$ d^{-1} [7,27,32,37,38], in agreement with our $k_{bio,AS}$ result.

For the bioaugmented AS, test A4 – with acetate supplementation, values of measured SMX concentration data plotted as a function of time elapsed show comparably high biotransformation rate in

364 the first 4-6 hours (when primary substrate was available), followed by lower removal rate during the
365 remaining time (following primary substrate depletion) (Fig. 2d). By using the obtained parameters $k_{bio,AS}$,
366 q_{bio_AS} from the A1 test, $k_{bio,PR1}$ (56.20 ± 3.70 L gTSS d^{-1}) from our previous study [14], fitting of measured
367 data in the substrate depletion phase using cometabolic model as described in Table 2 resulted in
368 estimation of $q_{bio,PR1}$ = 528.39 ± 6.78 L $gTSS^{-1}$ d^{-1}. This $q_{bio,PR1}$ value is consistent with rate constants
369 ($k_{bio,PR1}$ = 445.6 – 570.1 L $gTSS^{-1}$ d^{-1}) previously obtained with PR1 when acetate was supplemented as
370 a biogenic substrate to enhance the biotranforamtion rate of SMX in pure culture biodegradation tests
371 [14]. Higher biotransformation kinetics of SMX by PR1 ($q_{bio,\ PR1}$ and $k_{bio,\ PR1}$), compared to the
372 retransformation kinetics of the two human conjugates (k_{Dec}), likely lead to the observation of no increase
373 in SMX concentration in bioaugmented AS tests (A3 and A4), differently than what was observed in non-
374 bioaugmented AS tests (A1 and A2).
375 In general, two different kinetic rates of the removal of SMX are obtained for AS as well as for *A.*
376 *denitrificans* PR1: a fast rate q_{bio} when primary substrate was available and a slower rate k_{bio} when
377 primary substrate was depleted. These results can likely explain the two patterns of SMX
378 biotransformation observed in bioaugmented batch tests A3 and A4 (Fig. 2c and 2d). As a result of
379 cometabolism, SMX removal was enhanced in the presence of primary substrates (as characterized by
380 q_{bio}), with a subsequent decrease of biotransformation kinetics upon primary substrate limitation
381 (characterized by the k_{bio}) at the end of the A3 and A4 tests (Fig. 2c and 2d).
382 Also, the significant differences between k_{bio} and q_{bio} imply that growth substrates (readily biodegradable
383 substrates) availability can substantially impact the removal of SMX as a result of cometabolism. In fact,
384 typically present in wastewater at very low concentrations (ng L^{-1} to µg L^{-1}), micropollutants are unable
385 to support cell replication and primary substrates (e.g. readily biodegradable carbon sources or
386 ammonium) are essential for biomass growth and to induce enzymes for assimilation or co-factors for
387 biotransformation [39]. As wastewater is a complex medium where not only micropollutants but also
388 organic matter and nutrients are present, which could be degraded simultaneously by AS, cometabolism
389 kinetics could be suitable to predict the behavior of micropollutants in real WWTPs.
390 In addition, the SMX biotransformation rate constants by *A. denitrificans* PR1, e.g. $q_{bio,PR1}$ in the presence

391 and $k_{bio,PR1}$ in the absence of growth substrates, are three and two orders of magnitude higher, respectively,
392 than that estimated for AS, confirming a specialized biotransformation capability by PR1 in comparison
393 to the mixed AS community. Thus, bioaugmentation of AS with PR1 substantially enhanced the
394 biotransformation rate of SMX.

395 *Kinetics of SMX biotransformation under anoxic conditions*

396 SMX retransformation and removal under anoxic conditions can be predicted using pseudo-first order
397 kinetics (processes (7) – (9)), thereby allowing for the estimation of $k_{bio,Ax}$ (L $gTSS^{-1}$ d^{-1}). In Fig. 4a-b,
398 simulated and corresponding measured concentrations of the three compounds in An1 and An2 batch
399 experiments are plotted. High R^2 values (≥0.98) were obtained, and measured concentrations always fell
400 within the 95% confidence interval (Fig. S5, SI), indicating that the pseudo-first order model was able
401 to predict the fates of SMX and the two human conjugates obtained in the anoxic experiments. The
402 estimated $k_{bio,Ax}$ for SMX biotransformation are 13.57 ± 2.10 and 0 L $gTSS^{-1}$ d^{-1} for *A. denitrificans* PR1
403 and AS, respectively (Table 4). The latter value is in contrast with other studies. Plósz et al. [7] obtained
404 a SMX biotransformation rate constant of 0.41 L $gTSS^{-1}$ d^{-1} under anoxic conditions with AS. In other
405 studies, values of 0.1 and 0.05 L $gTSS^{-1}$ d^{-1} were reported for SMX biotransformation rate constants of
406 heterotrophic denitrification and autotrophic denitrification, respectively [32]. Torresi et al. [34]
407 reported a rate constant k_{bio} of 0.1 ± 0.1 L $gTSS^{-1}$ d^{-1} and q_{bio} of 1.7 and 3.2 for SMX biotransformation
408 in a post-denitrification MBBR system dosed with methanol and ethanol, respectively. The obtained
409 results suggest that, upon bioaugmentation to AS, PR1 could also be able to degrade SMX under anoxic
410 conditions but at a significantly lower rate as compared to aerobic conditions – decreasing by 4-fold in
411 terms of the rate constant ($k_{bio,PR1}$) under anoxic conditions as compared to aerobic conditions (Table 4).

412 In the current study, we also provided a detailed description of SMX removal in AS processes when
413 assessing the biotransformation of the parent compound and the deconjugation of the two major human
414 conjugates (Ac-SMX and SMX-Glu) back to SMX. SMX formation from the deconjugation of the two
415 human conjugates was experimentally observed and comfirmed by model-based predictions. Ac-SMX
416 and SMX-Glu were detected at levels that are comparable to the SMX concentrations in WWTPs [6,40]

417 (see also Table S3, SI). Significant retransformation of SMX can take place in WWTPs at a higher rate
418 compared to its removal rate (Table 4), resulting in the sometimes negative or varied SMX removal that
419 have been observed in many studies. It implies that deconjugation of human conjugates should be taken
420 into account to thoroughly understand the fate and removal of SMX during wastewater treatment.

421 In general, the results of these tests highlight the potential application of *A. denitrificans* PR1 for
422 bioaugmentation for SMX removal in WWTPs. One criterion for a successful bioaugmentation is the
423 metabolically active inoculum of a microorganism or consortium. Inability of the inoculated strains to
424 degrade the xenobiotic chemicals once augmented into AS has been reported [41]. One explanation
425 given for such failure in bioaugmentation was the presence of alternative readily biodegradable
426 substrates [41]. In our experiments, enhancement of SMX biotransformation upon bioaugmentation of
427 AS with PR1 was observed. Upon bioaugmentation of AS with *A. denitrificans*, without lag phase, a
428 fast biotransformation of SMX was observed at rates similar to those obtained in pure culture
429 biodegradation tests when acetate was supplemented as biogenic substrate. In addition, the SMX
430 reaction rate constant and cometabolic biotransformation rate of PR1 were about two orders of
431 magnitude higher than the kinetics of AS, regardless of the presence of additional acetate. The fact that
432 the strain was able to use the complex substrates present in real wastewater to stimulate the activity and
433 provide energy for growth and maintenance, suggests that PR1 has a great potential to survive in AS
434 communities upon bioaugmentation. Overall, bioaugmentation with PR1 appears to be a feasible
435 solution for enhancing SMX removal in wastewater, while further studies should focus on long-term
436 biotransformation activity and stability of the bioaugmentation strain in wastewater systems in order to
437 make bioaugmentation applicable.

438 ***3.4. Model validation***

439 The models for retransformation of SMX from the two human conjugates and cometabolic
440 biotransformation of SMX were validated using the two sets of experimental results, A2 and A3, for the
441 non-bioaugmented and bioaugmented cases, respectively (Fig. 2b and 2c). The set of estimated parameter
442 values ($k_{Dec,N4}$, $k_{Dec,Glu}$, $k_{bio,AS}$, $q_{bio,AS}$, $k_{bio,PR1}$, $q_{bio,PR1}$) was used to test the capability of the proposed models

443 to predict the behaviour of the three compounds (SMX, Ac-SMX and SMX-Glu) in the reactors A2 and
444 A3, providing measured data independent from those used for model identification. Measured and
445 predicted concentrations were compared and R^2 was calculated to determine the extent of correlation.
446 Good agreement between the experimental data and model simulations could be observed with high R^2
447 ($\geq$0.95, Table S4). This indicates the applicability of the model towards the prediction of the fate of SMX
448 and human metabolite biotransformation by both AS and PR1 (Fig. 2b and 2c), even in the presence of
449 an externally dosed carbon source (in this case acetate).

450 As cometabolic biotransformation depend on the readily biodegradable growth substrates, S_S (mgCOD
451 L^{-1}), the addition of acetate to AS would affect the biotransformation of SMX. From our previous study
452 [14] as well as the modelling result of the bioaugmentation test A4 (section 3.3.2), there is no doubt
453 that acetate is a biogenic substrate to enhance the SMX biotransformation by *A. denitrificans* PR1, i.e.
454 primary substrate for the cometabolism of SMX. For the non-bioaugmented activated sludge (A2), we
455 hypothesized above (section 3.2.1.2) that there is no enhanced effects of acetate on the biotransformation
456 of SMX by AS. To test this hypothesis, for modelling of SMX biotransformation of non-bioaugmented
457 AS (batch A2 – with the supplementation of acetate), we tested, (i) both acetate and other readily
458 biodegradable substrates that are present in wastewater (expressed as sCOD); (ii) only readily
459 biodegradable substrates that are present in wastewater (expressed as sCOD) were considered as the
460 primary substrates (S_S) in the cometabolic model to enhance the SMX biotransformation by AS.
461 However, only the latter option gave good fitting between measured and model-based prediction (Fig.
462 S7, SI vs. Fig. 2b), suggesting that the readily biodegradable substrates that are present in wastewater
463 (expressed as sCOD) acted and were sufficient as primary substrates for the cometabolism of SMX by
464 AS. Acetate was measured in the wastewater and it was typically below 7 mg/L, therefore the
465 microorganisms were probably not particularly adapted to it. Mũller et al. [33] observed that SMX
466 cometabolism with acetate by AS occurred only after a sufficient adaptation time, meaning that the
467 supplementation of additional acetate might have still enhanced the SMX further if sufficient adaptation
468 time was allowed, although little is known about which easily biodegradable compounds are used as
469 primary substrates by the AS community. Although the R^2 calculated for the SMX in the A2 test (Fig.

2b) was equal to 0.79, the difference between measured and predicted SMX concentrations were still within the standard deviations of the measured concentrations and falls in between confidence interval boundaries (Fig. S3), making the cometabolic model term still relevant.

Model calibration and validation results revealed that the applied models could predict accurately the fate of SMX, Ac-SMX and SMX-Glu. Kinetic parameter values describing the biotransformation of SMX, Ac-SMX and SMX-Glu under aerobic and anoxic conditions could therefore be implemented in AS models linking organic carbon removal (heterotrophic activity) and xenobiotic biotransformation to predict the fate of SMX in WWTPs. Overall, modelling is fundamental to understand the kinetics and the contribution of different members in bioaugmented AS communities with respect to xenobiotic biodegradation. Thus, combining modeling and experimental data offers the opportunity for a thorough understanding of elimination mechanisms of micropollutants in WWTPs to facilitate optimization of wastewater treatment processes and reduce emissions of xenobiotics.

4. Conclusions

In this study, six different batch tests with non-bioaugmented and bio-augmented (with *A. denitrificans* PR1) AS, operated under different redox conditions and primary substrate addition levels, were used for in-depth assessment of SMX removal in combination with its two main human conjugates (Ac-SMX and SMX-Glu). The extent of SMX removal varied depending on the experimental conditions, and process models were used for interpretation of experimental results and determination of biotransformation kinetics. The results from the experimental and model-based predictions show that the conversion of Ac-SMX and SMX-Glu back to parent SMX was confirmed in activated sludge, whereby deconjugation in non-bioaugmented AS was significantly faster than SMX biotransformation. This likely explains the previously observed variability of SMX removal efficiencies, including net formation of SMX, in full-scale biological WWTPs. The biotransformation of SMX and the deconjugation of Ac-SMX clearly depend on the redox conditions, with the highest removal occuring under aerobic conditions. Deconjugation of SMX-Glu was however independent of redox conditions. Also, cometabolic models were successfully used to predict the biological transformation kinetics of SMX in both bioaugmented

and non-bioaugmented reactors under different test conditions. Estimation of kinetic parameters allowed for the assessment of the success of the bioaugmentation strategy and for the identification of best conditions for its applicability. Furthermore, estimated kinetic parameters obtained from this study could be integrated in AS models to predict the fate of SMX during the biological treatment in WWTPs. Overall, bioaugmentation of AS with *A. denitrificans* strain PR1 led to enhanced (100-fold) biotransformation kinetics of SMX compared to the non-bioaugmented AS, within a complex carbon environment found at a WWTP without an addition of another C-source (acetate) as specific substrate for the biotransformation of SMX. These results prospect the use of *A. denitrificans* PR1 for bioaugmentation as a feasible and efficient option to improve SMX elimination in WWTPs.

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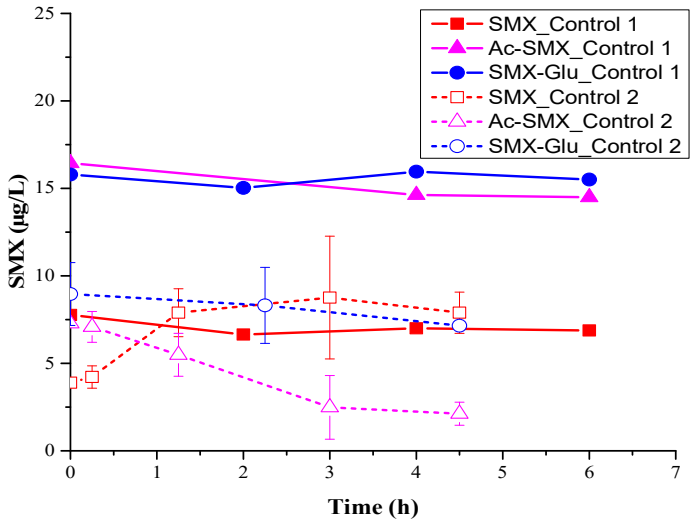

Fig. 1. Measured concentrations of SMX, Ac-SMX, and SMX-Glu as a function of time for the control batch tests, i.e. control 1-with Milli-Q water (continuous lines), control 2-with NaN_3 (dashed lines) as an inhibitor. Error bars indicate the standard deviations for duplicates

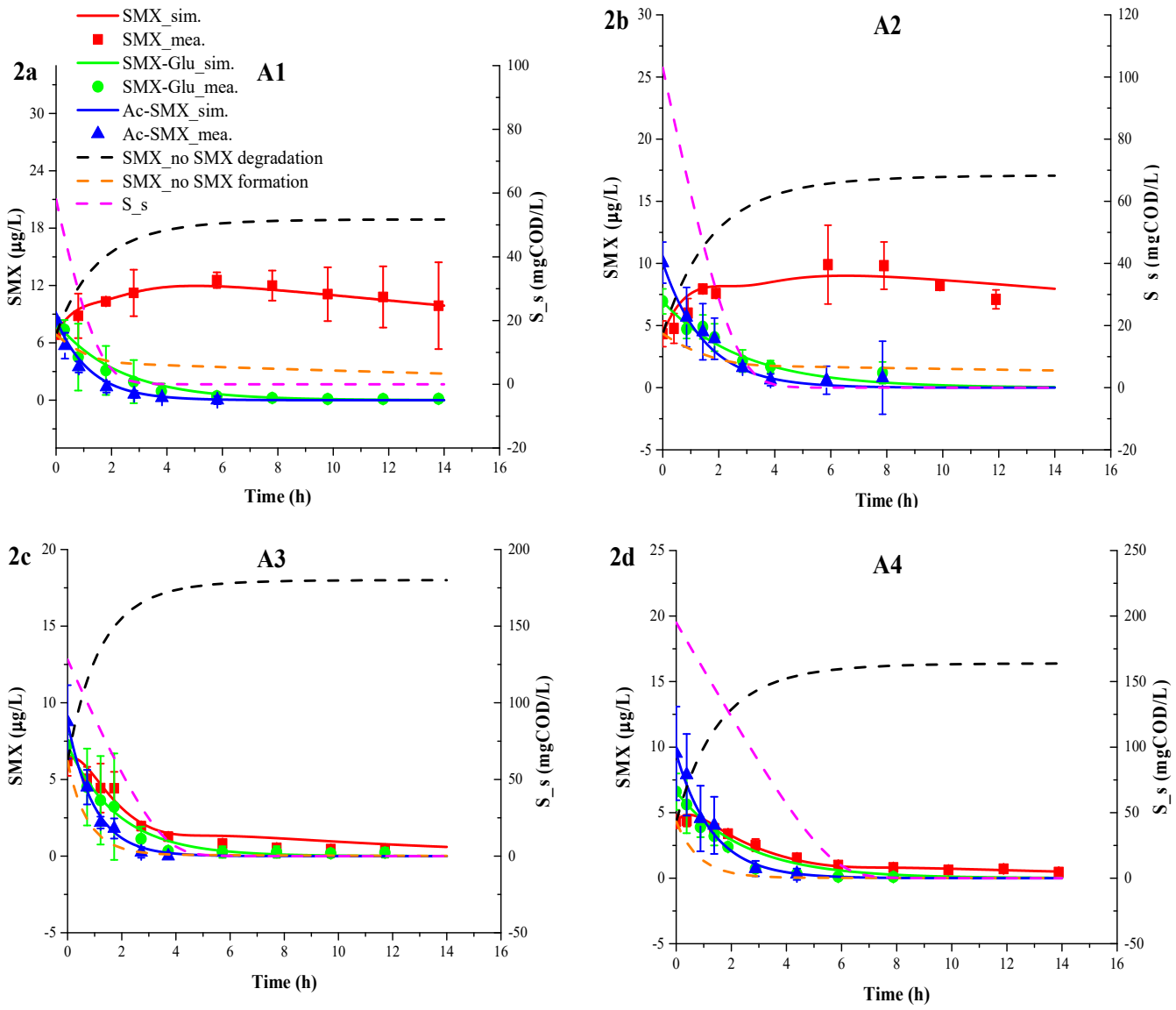

Fig. 2. Illustration of measured concentrations of SMX, Ac-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time for aerobic batch tests (A1): non-bioaugmented AS test (2a); (A2): non-bioaugmented AS test with supplementation of acetate as additional C-source (2b); (A3): bioaugmented AS with *A. denitrificans* PR1 test (2c); and (A4): bioaugmented AS with *A. denitrificans* PR1 supplemented with acetate test (2d). Orange dashed lines represent the SMX simulation if no SMX formation from the retransformation of Ac-SMX, and SMX-Glu. Black dashed lines represent the SMX simulation when all of Ac-SMX and SMX-Glu are converted back to parent SMX, but no SMX biodegraded. Error bars indicate the standard deviations for duplicates.

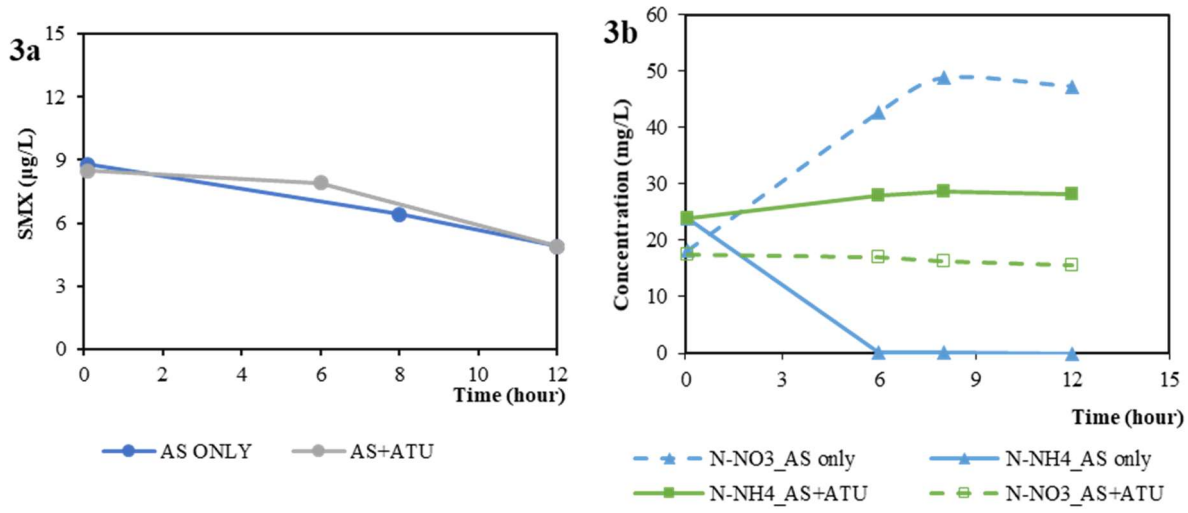

Fig. 3. Effect of ATU inhibition on removal of SMX (3a) and nitrogen (3b)

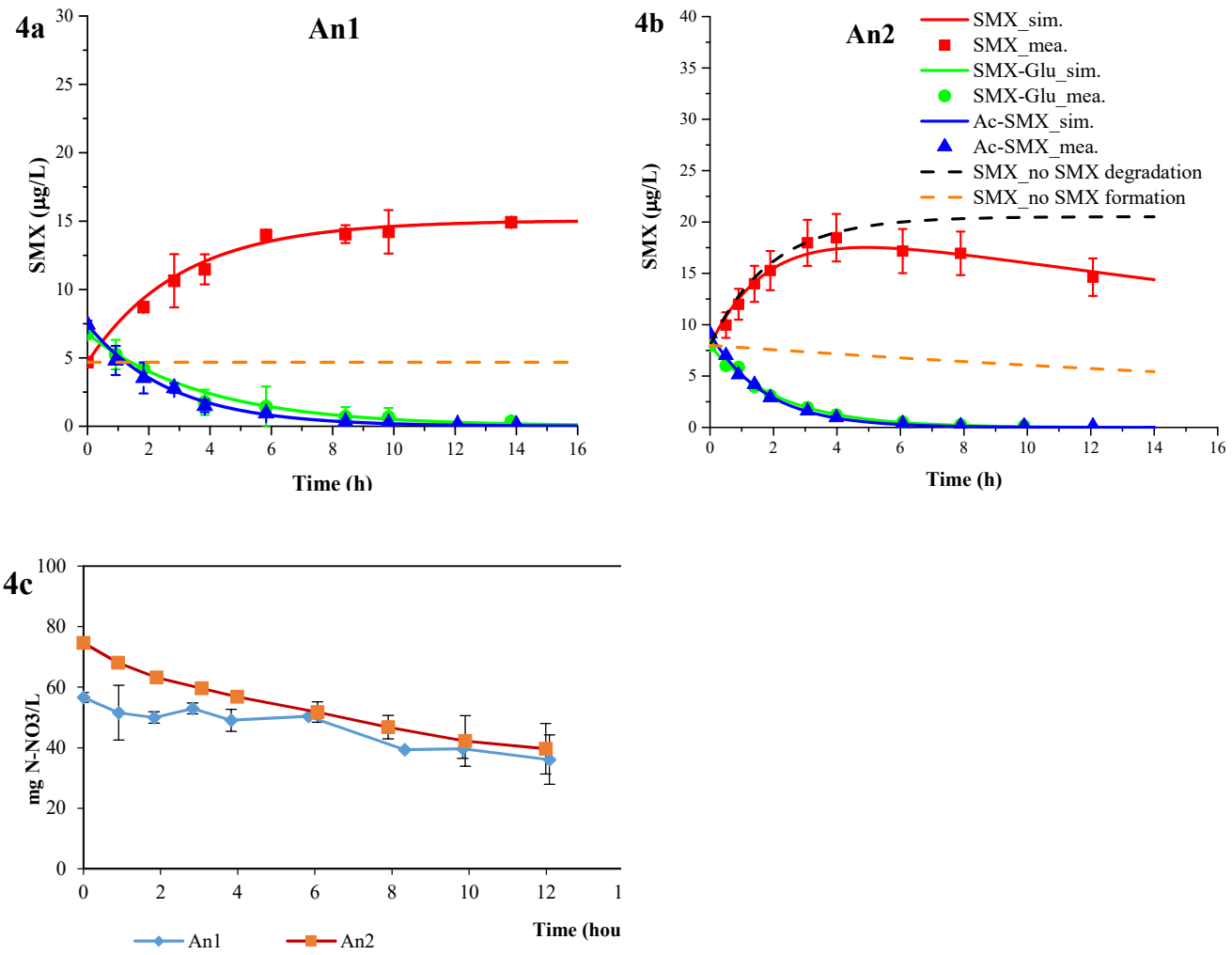

Fig. 4. Illustration of measured concentrations of SMX, N4-acetyl-SMX, and SMX-N1-Glucuronide (markers) and simulated (lines) as a function of time under anoxic conditions (An1): non-bioaugmented AS (4a); (An2): bioaugmented AS with *A. denitrificans* strain PR1 (4b). Orange dashed lines represent the SMX simulation if no SMX is formed from the retransformation of Ac-SMX, and SMX-Glu. Black dashed lines represent the SMX simulation when all of the Ac-SMX and SMX-Glu are converted back to SMX, but no SMX is biodegraded. Nitrate consumption for the batch tests is also shown (4c). Error bars indicate the standard deviations for duplicates.

Table 4. Model parameters and information of estimated kinetics for the biotransformations of SMX and the two human conjugates by activated sludge and *A. denitrificans* PR1 (PR1). Values in brackets indicate literature references.

Symbol	Definition	Unit	Compound: SMX-Glu		Compound: N4--SMX	
			AS	PR1	AS	PR1
			Aerobic			
$k_{Dec,Ox}$	Aerobic biotransformation rate coefficient for the human conjugates, C_{CJ}	L g TSS^{-1} d^{-1}	4.76 ± 0.38 (n.a)	-	8.9 ± 0.53 (5.9-7.6 [36]) (6.8[7])	-
$k_{bio,Ox}$	Aerobic biotransformation rate coefficient for the parent compound, C_{LI}	L g TSS^{-1} d^{-1}	-	-	-	-
$q_{bio,Ox}$	Aerobic cometabolic-biotransformation rate constant for parent compound, C_{LI}	L g TSS^{-1} d^{-1}	-	-	-	-
			Anoxic			
$k_{Dec,Ax}$	Anoxic biotransformation rate coefficient for the human conjugates, C_{CJ}	L g TSS^{-1} d^{-1}	4.74 ± 0.31 (n.a)	-	**5.30 ± 0.21** (7.9[13])	-

$k_{bio,Ax}$	Anoxic biotransformation rate coefficient for the parent compound, C_{LI}	L g TSS^{-1} d^{-1}	-	-	-	-

n.a.: not available

Supplementary Material Section

Bioaugmentation of activated sludge with *Achromobacter denitrificans* PR1 for enhancing the biotransformation of sulfamethoxazole and its human conjugates in real wastewater: Kinetic tests and modelling

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(14 pages, 5 Tables, 8 Figures)

S1. Sample preparation and analytical methods for the concentration of sulfamethoxazole, the two human metabolites and biodegradation metabolites with HPLC-LC-MS/MS

Sample preparation

The following SPE procedure was based on a previously published method for the analysis of sulfonamides in natural waters [1]. The sample was filtered through nylon syringe filter 0.2 µm (Whatman). The supernatant was stored in 10 mL glass vials until analysis (within 2 weeks). Before performing SPE, the sample aliquot was added with Na_2EDTA solution as a complexing agent, and was spiked with surrogate standards d4-N_4-acetyl-sulfamethoxazole, sulfamethoxazole-d4-N_1-glucuronide, sulfamethoxazole-d4 each at 500 ng/L, adjusted to pH = 3. Isotope labelled compounds were used to correct for any losses that may have occurred during SPE and quantify the compounds while accounting for matrix effects inherent to wastewater samples. Analytes were extracted using the hydrophilic-lipophilic balance OASIS HLB catridge (6 mL, 200 mg) from Waters (Millford, MA). The cartridge was pre-conditioned with 6 mL of MeOH, followed by 3 mL of acidified metanol (0.1% formic acid in HPLC grade methanol, v/v), and then 2 x 6 mL of MilliQ-water. After that, samples were extracted through the HLB cartridges at a flow rate of ~5 mL/min using a 20-position vacuum manifold (Waters). After extraction, the cartridge was rinsed with 2 x 6 mL of MilliQ-water and vacuum-dried for ~5 min. The retained analytes were subsequently eluted with 4 x 2 mL of acidified methanol (50 mM formic acid) into a glass test tube. The SPE eluent was evaporated to dryness under a gentle flow of nitrogen and finally reconstituted to 500 µL in a solvent mixture of MilliQ-water:methanol (9:1). The extract was transferred to an amber autosampler vial, and stored at -20^{o}C until LC-MS/MS analysis, which was carried out the day after.

Analytical methods

The concentration of SMX and their metabolites were monitored by using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. This equipment is equipped with a binary pump, an automatic injector and a thermostatted column compartment coupled to a Mass Spectrometer TSQ Endura triple quadrupole model, from Thermo Scientific. The separation was performed on a reversed-phase column (Acquity BEH C18 (2,1 x 50 mm, 1,7 µm), Waters)) at 40^{o}C using an injection volume of 20 µL. The mobile phase consisted of water:formic acid 0.5% v/v supplemented with 0.01 mM ammonium acetate (A): methanol (B) at a flow rate of 0.30 mL/min and the eluting conditions applied consisted of 2 min at 5% of B; 2 min at 20% of B, 2 min at 50% of B followed by 2 more minutes at 70% of B then a linear gradient up to 90% of B for 2 min before finally reduced to 5 % of B for the last 3 min.

Triple quadrupole operating conditions were optimized in order to work in multiple reaction monitoring mode (MRM). The optimization was based on the selection of ionization mode, optimum collision energy (eV), cone voltage.

Ionization was achieved by positive electron spray ionization (ESI), using a spray voltage of 4 kV situated at a 90° angle to the entrance. Drying gas temperature was set as 350°C, nebulizer pressure (N_2) as 22 psi and drying gas flow rate as 11 L/min to achieve the highest sensitivity. Ultra high-purity Argon (Ar) was used as collision gas. High purity nitrogen was used as sheath, auxiliary and sweep gas.

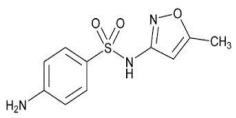
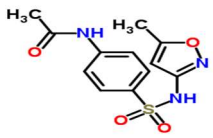
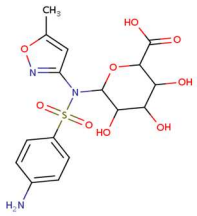
MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table S1. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

XCalibur software (version 4.1) was used for data acquisition and processing.

Table S5. MS/MS parameters for the analysis of target analytes by MRM positive ionization mode

Target compounds	**R_t (min)**	**Precursor ion $[M+H]^+$**	**MRM1**		**MRM2**	
			Collision energy (eV)	**Production ion**	**Collision Energy (eV)**	**Production ion**
Sulfamethoxazole	5.84	254.1	15	156	20	92
N_4-acetyl-sulfamethoxazole	6.81	296.3	25	134.1	18	198.1
sulfamethoxazole-N_1-glucuronide	4.93	430.3	10	254.3	30	156.1
d4-sulfamethoxazole	5.80	258.2	15	160.1	25	96.1
d4-N_4-acetyl-sulfamethoxazole	6.82	300.3	25	138.2	18	202.2
sulfamethoxazole-d4-N_1-glucuronide	4.87	434.3	12	258.3	30	160.1
3-amino-5-methylisoxazole	0.89	99.1	10	99.2>72	12	99>44

Table S6. Target pharmaceuticals and transformation products under investigation: structure, properties, and wastewater concentration (K_{ow} – octanol–water partition coefficient; K_d – solid–liquid partition coefficient; pK_a–acid dissociation constant; TP – transformation product; N.F – not found).

Chemical	Structure	Use	Log K_{ow}	Log K_d	pK_a	WWTP primary effluent incidence concentration (µg L^{-1})
Sulfamethoxazole		Antibiotic	0.89[2]	2.4[3]	pK_{a1} = 1.8[4] pK_{a2} = 5.7[4]	0.87 ± 0.75
Ac-acetyl-sulfamethoxazole		Antibiotic TP	N.F	N.F	5.6 ± 0.5[4]	0.98 ± 0.2
Sulfamethoxazole-N_1-glucuronide		Antibiotic TP	1.21	N.F	2.7 ± 0.5[4]	n.d.

n.d. : not determined

S2. Chelas Wastewater Treatment Plant (Lisbon, Portugal)

Municpal WWTP Chelas was designed to receive about 52500 m^3 of wastewater per day, with a capacity of 211000 population equivalents (PE). The WWTP comprises various treatment processes such as a pre-treatment, primary treatment, biological treatment (anoxic-aerobic process), tertiary treatment (sand filtration → UV) and sludge treatment. The biological treatment was designed for nitrogen removal with a pre-denitrificcation process and operated at hydraulic retention time (HRT) of 2 hours. Biogas produced from the anaerobic digestion process of sludge treatment is used as energy to lower the plant operational cost. Characteristics of the primary effluent wastewater are mentioned in Table S3. The average treatment performance of Chelas WWTP was 90% removal of $N\text{-}NH4^+$, 60% removal of $N\text{-}NO_3^-$.

Table S7. Primary effluent wastewater characteristics

	TCOD ($mg\ L^{-1}$) (n=23)	sCOD ($mg\ L^{-1}$) (n=23)	BOD_5 ($mg\ L^{-1}$) (n=13)	$N\text{-}NH_4^+$ ($mg\ L^{-1}$) (n=76)	SMX ($\mu g\ L^{-1}$) (n=3)	Ac-SMX ($\mu g\ L^{-1}$) (n=3)
Range	126-500	50-139	33-143	40-53	0.52 – 1.73	0.78 -1.17
Mean ± std	258 ± 97	99 ± 22	91 ± 37	40 ± 8	0.87 ± 0.75	0.98 ± 0.20

TCOD: total COD; sCOD: soluble COD

S3. Model calibration procedure

For model calibration, the $k_{bio,AS}$ value was approximated based on process (2) (Table 2) using the tangent value of the linear regression line fitted to measured data obtained in the primary substrate limitation period (from 6 hours to 14 hours, after retransformation was completed and growth substrates were depleted (Fig. 2a). K_D values (shown in Table 2) were used to assess the sorption fraction, while the constant value X_{AS} in Table 2 represent activated sludge (AS) biomass concentration. Process (1), (4) and (6) (Table 2) allows estimation of $q_{bio,AS}$, using the $k_{bio,AS}$ value obtained above, the K_D value (shown in Table 2), and a constant value for X_{AS}.

Data obtained from the bioaugmented test A4 was used to determine the cometabolic biotransformation rate constant of *A. denitrificans* PR1, i.e. $q_{bio,PR1}$. Biotransformation of SMX in this experiment was attributed to the activity of both AS and the strain PR1 (Table 2, process (1), (4), (5) and (6)) and characterized by the biotransformation rate constants of AS ($k_{bio,AS}$ and $q_{bio,AS}$) and of PR1 ($k_{bio,PR1}$ and $q_{bio,PR1}$). Biotransformation kinetic associated with AS were previously estimated through calibration against A1 test results, while $k_{bio,PR1}$ was derived from our previous study under primary substrate limitation [5].

The biotransformation of SMX under anoxic conditions is predicted using pseudo-first order kinetics, thereby allowing for the estimation of the biotransformation rates $k_{bio,Ax}$ ($L\ gSS^{-1}\ d^{-1}$).

Experimental results from An1 were used to determine the retransformation rate, i.e. $k_{Dec,Ax}$ and SMX biotransformation kinetics of AS, i.e. $k_{bio,AS}$, under anoxic conditions using processes (7) and (8) (Table 2). The anoxic $k_{bio,AS}$ was then used as an input for predicting An2 results and estimating the biotransformation rate coefficient of strain PR1 ($k_{bio,PR1}$) using processes (7), (8) and (9) (Table 2).

S4. Standard deviation calculated for average samples

The standard deviations of the measured concentrations of the three target compounds, e.g. sulfamethoxazole, N_4-acetyl-sulfamethoxazole, and SMX-N_1-Glucuronide were calculated by STDEV function in Excel that uses the following formula:

$$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}} \quad \text{(Eq. S1)}$$

where x is the sample mean of the duplicate of two tests and n is the sample size, in this study n=2.

Table S8. Goodness of the fit (R2) of the models used in this study

Test	Compound	Biotransformation		Retransformation
		Pseudo-first order kinetic	Cometabolic enhancement kinetic	Pseudo-first order kinetic
A1	SMX	0.04	0.95	
	Ac-SMX			0.99
	SMX-Glu			0.98
A2	SMX	0.52	0.79	
	Ac-SMX			0.99
	SMX-Glu			0.98
A3	SMX	0.94	0.95	
	Ac-SMX			0.99
	SMX-Glu			0.98
A4	SMX	0.89	0.99	
	Ac-SMX			0.98
	SMX-Glu			0.94
An1	SMX	0.99		
	Ac-SMX			0.99
	SMX-Glu			0.99
An2	SMX	0.98		
	Ac-SMX			0.99
	SMX-Glu			0.99

Table S5. Parameters of the ASM model [6] **used and calibrated in this study**

Parameter	Definition	Values	Unit
μ_H	Specific growth rate of heterotrophs	Calibrated	day^{-1}
Y_H	Yield coefficient for heterotrophs	0.67	g cell COD formed (g COD oxidized)$^{-1}$

K_S	Saturation constant for substrate S_S	20	gCOD m^{-3}

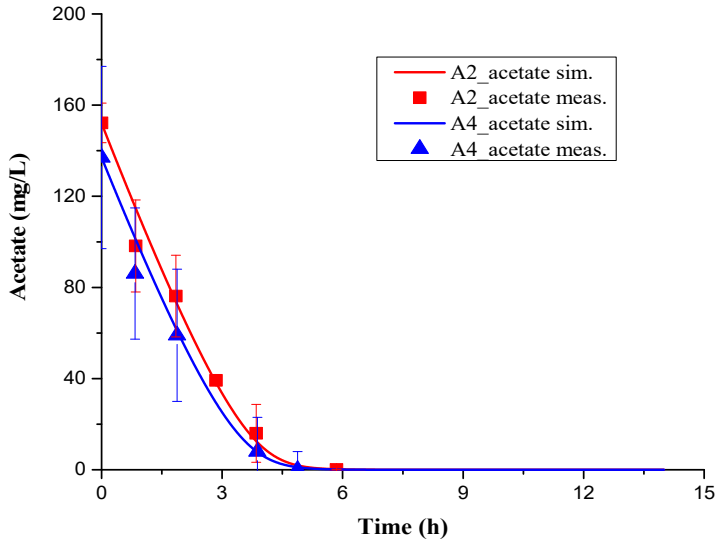


Fig. S5. Measured and simulated acetate, expressed as sCOD (mg/L) for aerobic batch tests (A1): non-bioaugmented AS with supplementation of acetate; and (A4): bioaugmented AS with supplementation of acetate. Error bars indicates standard deviation for duplicates

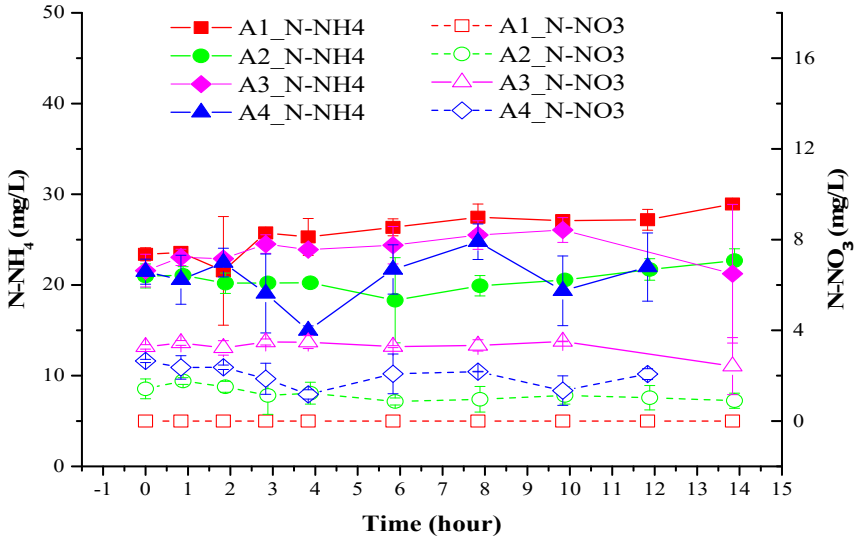


Fig. S2. Evolution of ammonium concentration for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* supplemented with acetate test. Error bars indicate standard deviations for duplicates.

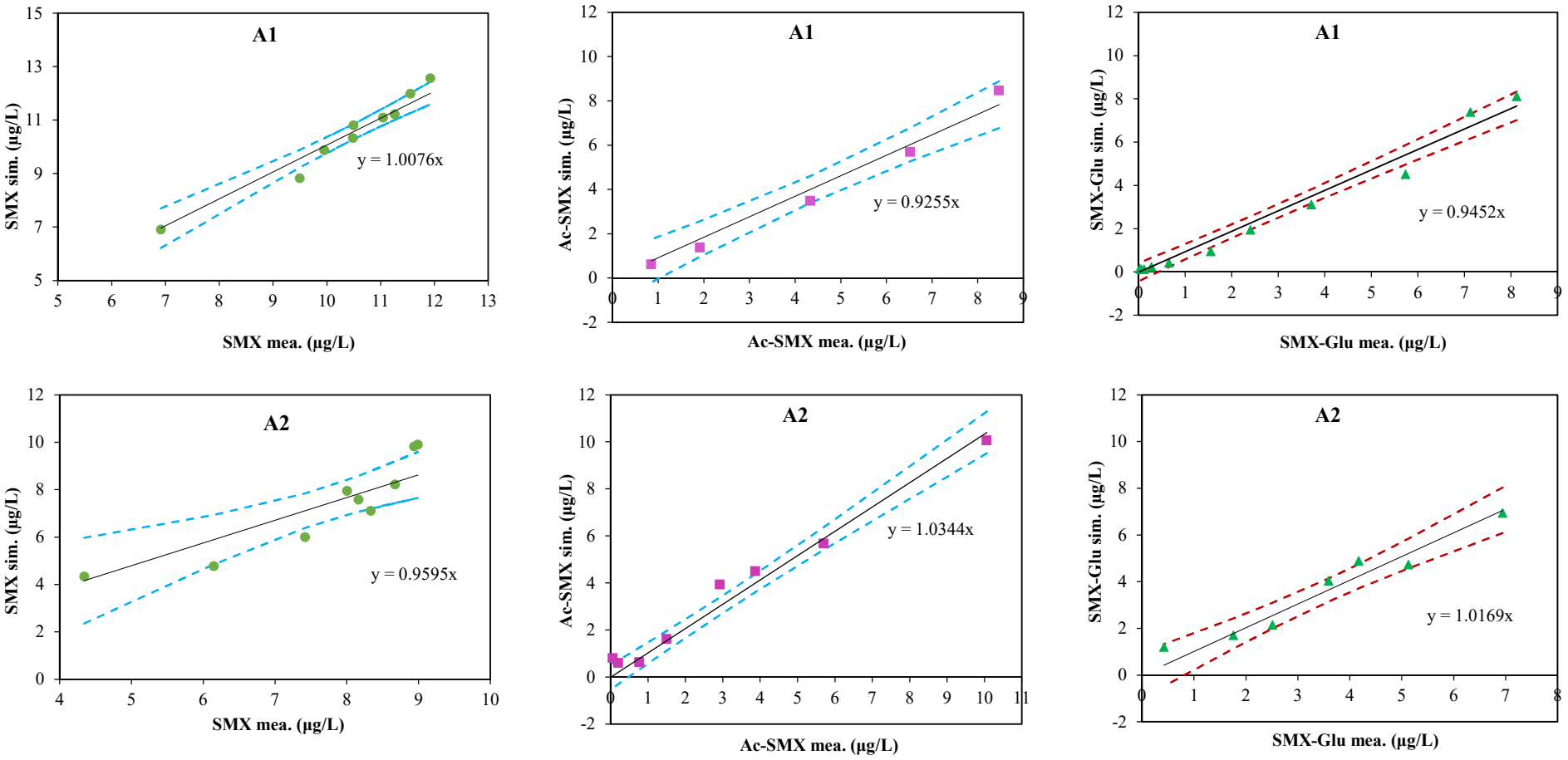


Fig. S3. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the non-bioaugmented aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplement of acetate as additional C-source. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

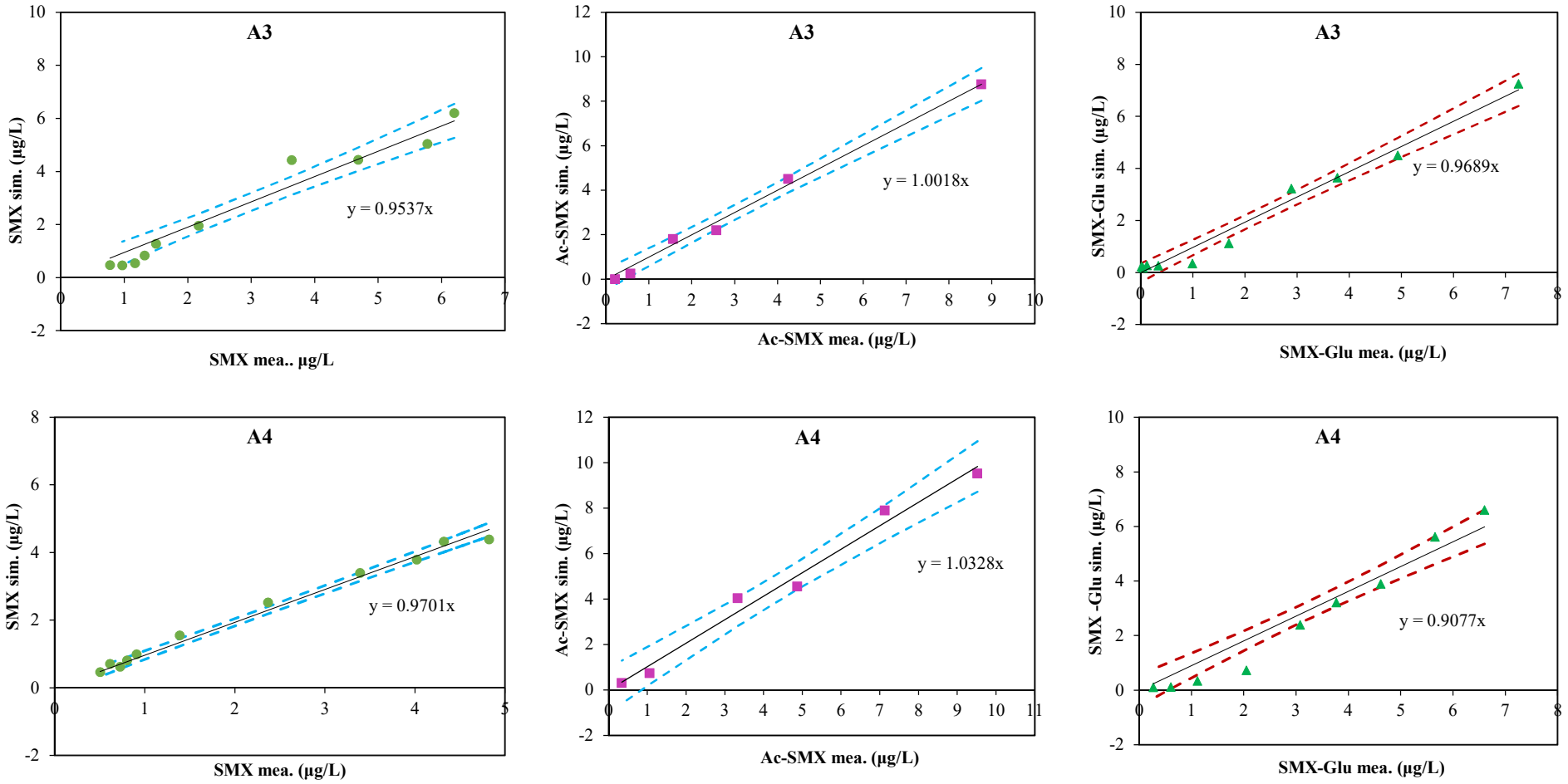


Fig. S4. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the bioaugmented aerobic batch tests (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* PR1 supplement with acetate test. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

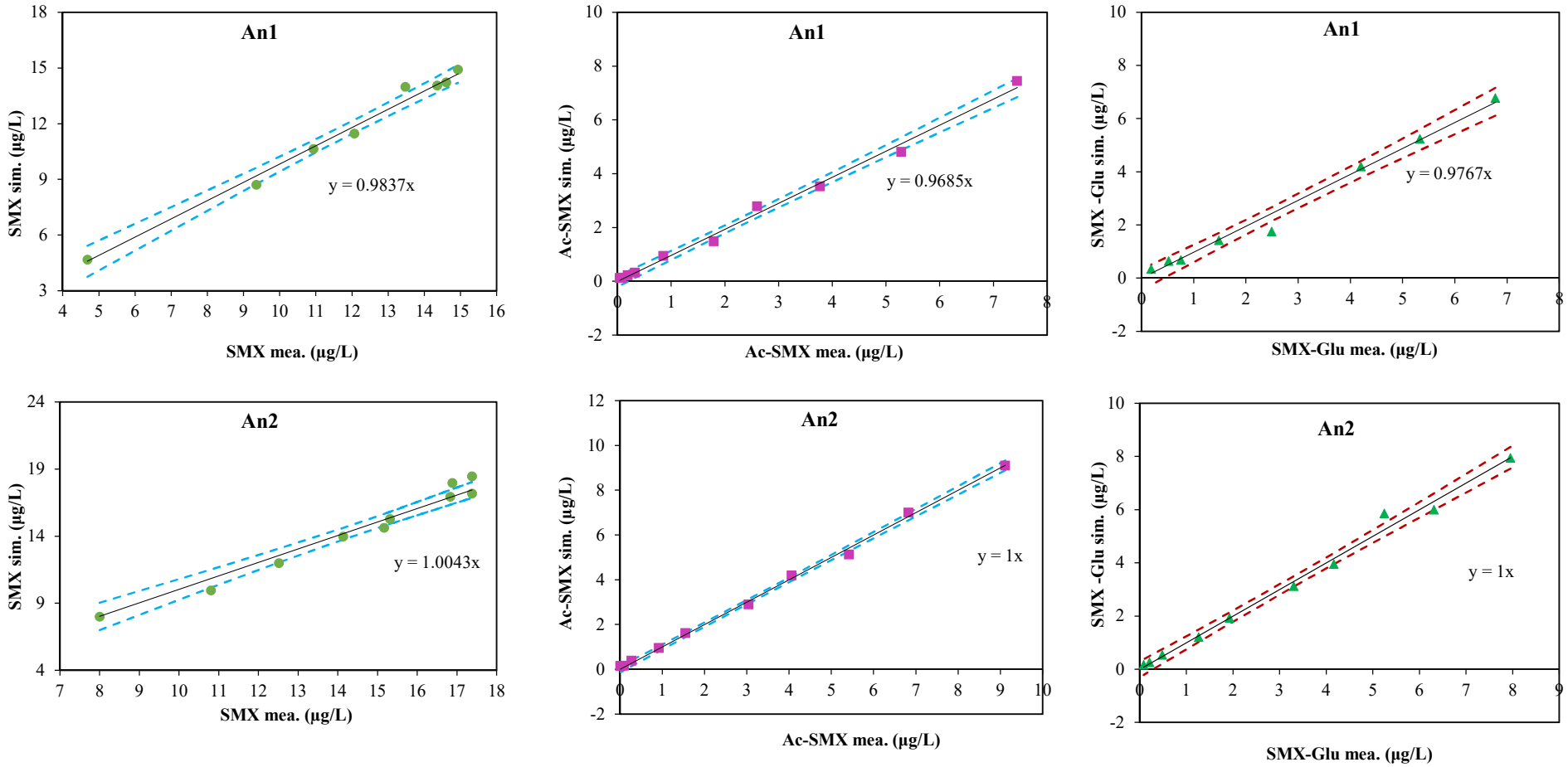
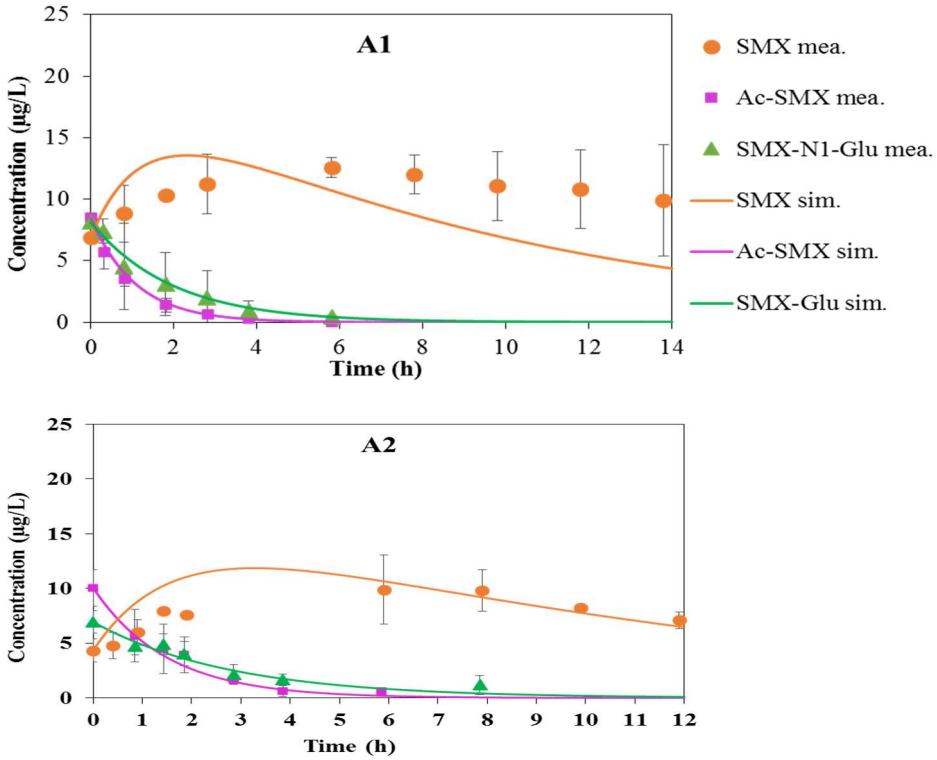


Fig. S5. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the anoxic batch tests (An1): non-bioaugmented activated sludge; (An2): bioaugmented activated sludge with *A. deninitrificans* PR1. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.


A1
Concentration (μg/L)
Time (h)
SMX mea.
Ac-SMX mea.
SMX-N1-Glu mea.
SMX sim.
Ac-SMX sim.
SMX-Glu sim.
A2
Concentration (μg/L)
Time (h)

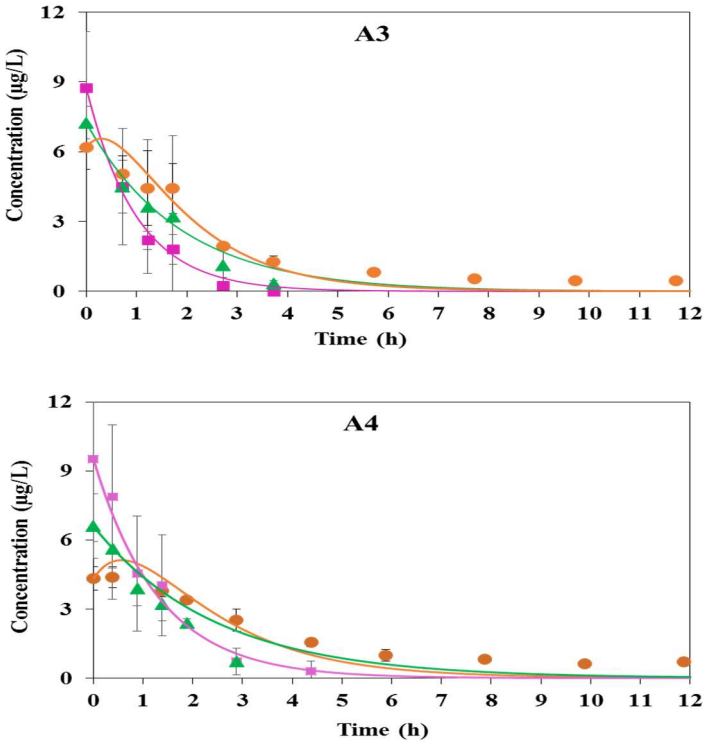


Fig. S6. Illustration of measured concentrations of SMX, Ac-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* PR1 supplemented with acetate test. Error bars indicate the standard deviations for duplicates

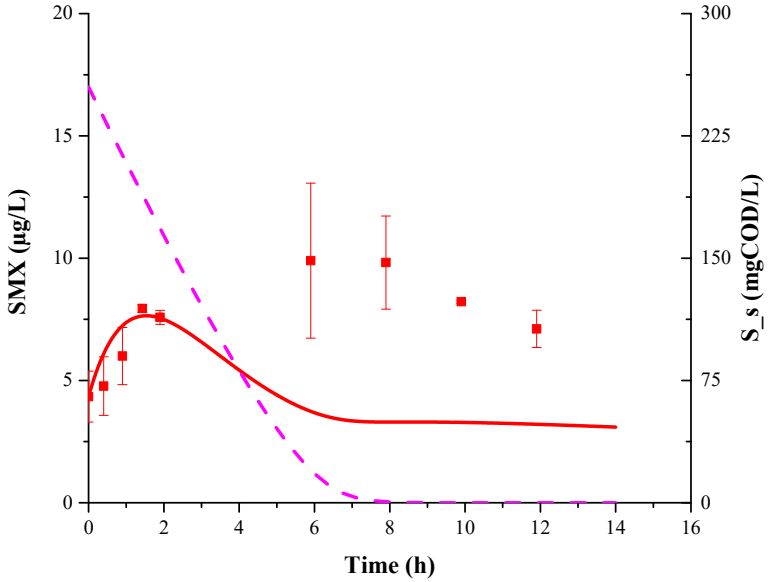


Fig. S7. Illustration of measured (makers) and simulated (continuous lines) concentrations of SMX as a function of time for aerobic batch tests (A2): non-bioaugmented AS test with supplementation of acetate as additional C-source, with both acetate and other readily biodegradable substrates that present in wastewater (expressed as sCOD) were considered as the primary substrates (S_S) in the cometabolic model to enhance the SMX biotransformation by AS

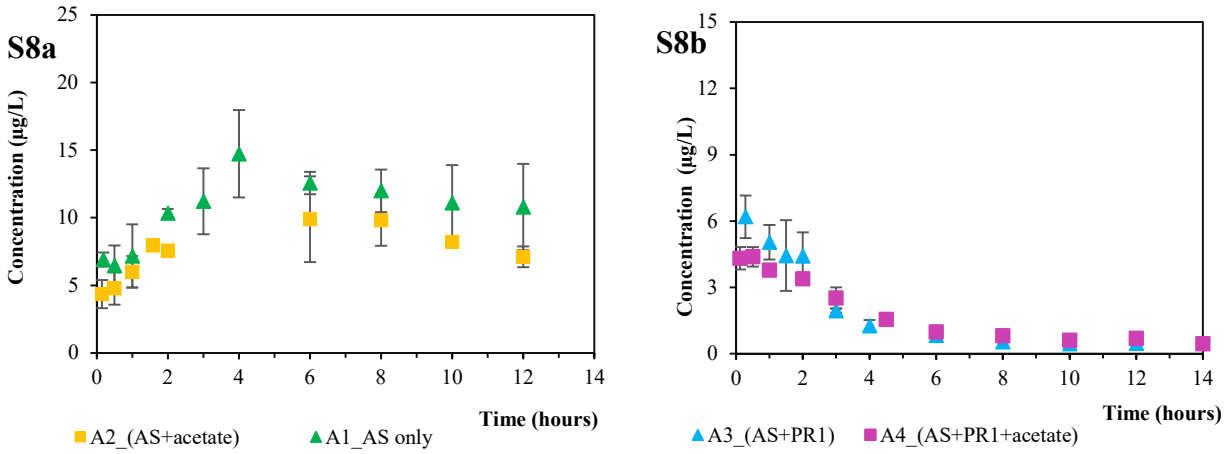


Fig. S8. Measured concentrations of SMX as a function of time for the batch tests of non-bioaugmented AS (S8a) and bio-augmented one (S8b), with and without the addition of acetate under aerobic conditions. Error bars indicate the standard deviations for duplicates.

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